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DTU Environment
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PhD Thesis
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The synopsis part of this thesis is available as a pdf-file for download from the DTU research database ORBIT: http://www.orbit.dtu.dk

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Preface

This PhD thesis, entitled "Unconventional biomasses as feedstocks for production of biofuels and succinic acid in a biorefinery concept", comprises the research carried out at the Department of Environmental Engineering, Technical University of Denmark from December 15, 2011 to December 14, 2014. Professor Irini Angelidaki and Senior Researcher Dimitar Karakashev were supervisor and co-supervisor, respectively.

The thesis is organized into two parts: the first part is a literature review of the thesis topic where the findings of the PhD project are put into context; the second part consists of the papers published in scientific journals listed below. These will be referred to in the text by their paper number written with the Roman numerals I-VI.

I Gunnarsson, I.B., Svensson, S.-E., Johansson, E., Karakashev, D., Angelidaki, I., 2014. Potential of Jerusalem artichoke (*Helianthus tuberosus* L.) as a biorefinery crop. *Industrial Crops and Products* 56, 231–240.

II Gunnarsson, I.B., Karakashev, D., Angelidaki, I., 2014. Succinic acid production by fermentation of Jerusalem artichoke tuber hydrolysate with *Actinobacillus succinogenes* 130Z. *Industrial Crops and Products* 62, 125–129.

III Gunnarsson, I.B., Morales, A.-M., Angelidaki, I., 2014. Utilization of CO_2 fixating bacterium *Actinobacillus succinogenes* 130Z for simultaneous biogas upgrading and bio-succinic acid production. *Environmental Science & Technology* 48, 12464–12468.

IV Gunnarsson, I.B., Kuglarz, M., Karakashev, D., Angelidaki, I., 2014. Thermochemical pretreatments for enhancing succinic acid production from industrial hemp (*Cannabis sativa* L.). Submitted.

V Kuglarz, M., Gunnarsson, I.B., Svensson, S.-E., Prade, T., Johansson, E., Angelidaki, I., 2014. Ethanol production from industrial hemp: effect of combined dilute acid/steam pretreatment and economic aspects. *Bioresource Technology* 163, 236–43.

VI Morales, A.-M., Gunnarsson, I.B., Fotidis, I.A., Vasilakou, E., Lyberatos, G., Angelidaki, I., 2014. *Laminaria digitata* as a potential carbon source for succinic acid and bioenergy production in a biorefinery perspective. Submitted.

In this online version of the thesis, the papers are not included but can be obtained from electronic article databases e.g. via www.orbit.dtu.dk or on request from DTU Environment, Technical University of Denmark, Miljøvej, Building 113, 2800 Kgs. Lyngby, Denmark, reception@env.dtu.dk.

In addition, the following publications, not included in this thesis, were also concluded during this PhD study:

> Gunnarsson, I.B., Morales, A.-M., Angelidaki, I. 2014. Methods for upgrading of a fuel gas and succinic acid production. Patent publication number WO/2014/188000.

Acknowledgements

Reflecting on the three years I spent as PhD student at DTU Environment, the first thing that comes to my mind is how much I enjoyed my time there and how much I appreciate the guidance and friendship of the brilliant people working there.

I would like to thank my supervisor Professor Irini Angelidaki for her guidance during these three years. Also, for giving me various responsibilities and trusting me with a variety of tasks that in my opinion have added another dimension to my PhD project.

Likewise, I thank my co-supervisor, senior researcher Dimitar Karakashev for inspiring me when needed, for his supervision and support.

Additionally, I would like to give special thanks to my friend Merlin Alvarado-Morales, who I collaborated extensively with.

I would also like to give thanks to: Goncalo Silva Marinho, Mike Podevin, Jonathan Van Wagenen, Ana Tomás, Ioannis Fotidis, Susan Holdt, Panagiotis Kougias, Kanokwan Boe, Davide De Francisci, Yifeng Zhang, Laura Treu, Gang Luo, Panagiotis Kougias, Mariusz Kuglarz, Eva Johansson, Sven-Erik Svensson, Jens Sørensen, Hector Garcia and Morten Andreasen.

Last but not the least; I want to thank my whole family, especially my amazing wife and two beautiful sons for making me a better person and for always believing in me, and supporting me in everything I do.

Abstract

Biorefinery has the potential of displacing fossil fuels and oil-refinery based products. Within the biorefinery a palette of marketable commodities can be produced from biomass, including food, feed, biochemicals and biofuels. Which bioproducts are produced is largely dependent on the chemical composition of the specific biomass feedstock, as well as which pretreatment, saccharification, fermentation and extraction techniques are used. Furthermore, integrating biological processes into the biorefinery that effectively consume CO_2 will become increasingly important. Such process integration could significantly improve the sustainability indicators of the overall biorefinery process.

In this study, unconventional lignocellulosic- and aquatic biomasses were investigated as biorefinery feedstocks. The studied biomasses were Jerusalem artichoke, industrial hemp and macroalgae species *Laminaria digitata*. The chemical composition of biomasses was determined in order to demonstrate their biorefinery potential. Bioethanol and biogas along with succinic acid production were the explored bioconversion routes, while potential production of other compounds was also investigated.

Differences and changes in biomass composition and productivity of eleven different Jerusalem artichoke clones was examined at three harvest times. Yields of up to 35 t ha^{-1} of dry lignocellulose matter was reported, nonetheless the amount of cellulose in many cases was less than 50% of what was observed in e.g. hemp. However, the underground tubers which the plant produces, contained high amounts carbohydrates (≤88% of dry weight) and yielded up to 6 t ha^{-1} dry matter of additional carbohydrates. The carbohydrate content found in *L. digitata* was also shown to be exceptionally high (77.6% of dry weight) compared to other studies.

Diverse methods for pretreatment and saccharification of biomass were used depending on the type of biomass. *L. digitata* did not required any pretreatment before enzymatic hydrolysis other than milling and drying. Pretreatments using H_2SO_4, NaOH and H_2O_2 at different conditions were used to pretreat hemp prior to enzymatic hydrolysis, while Jerusalem artichoke tubers needed 0.2% H_2SO_4 in combination with heat-treatment as a direct hydrolysis method.

Bioethanol was produced from industrial hemp hydrolysates. Ethanol yields in the range of 74-92% of theoretical yield were reported, while ethanol

concentrations amounted up to 10.0 g L^{-1}. However, the production of succinic acid from this type of hydrolysate resulted in much higher product titer and substrate utilization compared to ethanol fermentation, partially because *A. succinogenes* is able to ferment both glucose and xylose into succinic acid.

Jerusalem artichoke tubers, industrial hemp and *L. digitata* all showed considerable potential as feedstock for succinic acid production. The maximum succinic acid production from the different feedstocks ranged between 21.9 and 47.4 g L^{-1}. The highest succinic acid titer was reached when fermenting Jerusalem artichoke hydrolysate, while the maximum succinic acid yield (86.5%) was reached when fermenting *L. digitata* hydrolysate. In the case of tuber biomass it was shown that tubers could be readily hydrolyzed without enzymes and fermented without any addition of nutrients, which clearly indicates that utilization of this feedstock could potentially lower the costs for succinic acid production.

The biochemical methane potential of *L. digitata*, post hydrolysis solid residue (PHSR) and fermentation broth after succinic acid fermentation was also determined. In a biorefinery, biogas production is important for energy recovery as well as for minimizing waste and generating an additional product in the form of fertilizer. Energy recovery of PHSR and fermentation broth through anaerobic digestion corresponded to 298 and 285 NmL CH_4 g^{-1} VS_{added}, respectively.

To further increase the integration of the different processes in the biorefinery concept, a novel biogas upgrading technology was developed. The approach was based on the CO_2 fixation abilities of *A. succinogenes* to simultaneously produce high purity CH_4 and succinic acid. The system was able to reach 95.4% CH_4 content, which is similar purity as commercial biogas upgrading technologies deliver.

Results obtained in this study constitute the first report for utilization of macroalgae, hemp and Jerusalem artichoke tuber biomass for fermentative succinic acid production. It was demonstrated that all biomasses are attractive biomass feedstocks for succinic acid production mainly due to their high carbohydrate content. A case study of a proposed macroalgae biorefinery concept highlighted the potential of post hydrolysis solid residue (PHSR) for the production of numerous additional products such as ω-3 and ω-6 fatty acids, biodiesel, protein, feed, biogas and fertilizer, thereby diversifying the biorefinery product portfolio.

Dansk sammenfatning

Et bioraffinaderi har potentiale til at fortrænge fossile brændstoffer og olie-baserede produkter. I et bioraffinaderi kan en række forskellige produkter fremstilles af biomasse, herunder fødevarer, foder, biokemikalier og biobrændstoffer. Hvilke produkter der produceres, er i høj grad afhængig af den kemiske sammensætning af den specifikke biomasse, samt hvilken forbehandling, saccharificeringsprocesser, gæring og udvindingsteknikker der anvendes. Desuden vil biologiske processer der integrerer effektivt forbrug af CO_2, med produktion af biokemikalier blive stadig vigtigere. En sådan procesintegration kan i væsentlig grad forbedre bæredygtigheden af de overordnede processer i et bioraffinaderi.

I denne undersøgelse blev ukonventionelle lignocellulære og akvatiske biomasser undersøgt som bioraffinaderi-biomasser. De undersøgte biomasser var jordsskokke, industrihamp og makroalgearten Fingertang. Den kemiske sammensætning af biomasserne blev bestemt for at demonstrere deres potentiale i et bioraffinaderi. Bioethanol, biogas og ravsyre var de undersøgte bioprodukter, mens potentiel produktion af andre forbindelser også blev undersøgt.

Forskelle og ændringer i biomassen sammensætning og produktivitet af elleve forskellige jordskokke-kloner blev undersøgt ved tre høsttidspunkter. Udbytte på op til 35 t ha^{-1} tør lignocellulose blev opnået, men mængden af cellulose var i mange tilfælde mindre end 50% af, hvad der blev observeret i fx hamp. Planternes rødder indeholdte høje mængder kulhydrater (≤88% af tørvægt) og gav op til 6 t ha^{-1} tørstof af yderligere kulhydrater. Kulhydratindholdet i *L. digitata* viste sig at være usædvanlig høj (77.6% af tørvægt).

Forskellige metoder til forbehandling og saccharificering af biomasse blev anvendt afhængig af typen af biomassen. *L. digitata* krævede ingen anden forbehandling end formaling og tørring før enzymatisk hydrolyse. Forbehandlinger med H_2SO_4, NaOH og H_2O_2 ved forskellige betingelser blev anvendt til at forbehandle hamp før enzymatisk hydrolyse, mens jordskokke-knolde blev behandlet med 0.2% H_2SO_4 i kombination med varmebehandling som en direkte hydrolysemetode.

Bioethanol blev produceret af industrihamp-hydrolysater. Ethanoludbytte i området 74-92% af det teoretiske udbytte blev opnået, mens ethanol koncentrationer var op til 10.0 g L^{-1}. Produktion af ravsyre fra denne type af

hydrolysat resulterede i meget højere koncentrationer og substratudnyttelse i forhold til ethanolfermentering, delvist fordi A. succinogenes er i stand til at fermentere både glucose og xylose til ravsyre.

Jordskokke-knolde, industrihamp og *L. digitata* viste alle stort potentiale som råvare til ravsyreproduktion. Den maksimale ravsyreproduktion varierede mellem 21.9 og 47.4 g L^{-1}. Den højeste ravsyrekoncentration blev opnået med gæring af jordskokke hydrolysat, mens det maksimale ravsyre udbytte (86.5%) blev opnået ved gæring af *L. digitata* hydrolysat. Med rodknolde-biomasse blev vist, at knolde let kunne hydrolyseres uden enzymer og fermenteres uden tilsætning af næringsstoffer, hvilket påviser, at anvendelsen af dette råmateriale potentielt kunne reducere omkostningerne for ravsyreproduktion.

Det biokemiske metanpotentiale for *L. digitata*, PHSR (e. post hydrolysis solid residue) og fermenteringsvæsken efter ravsyregæring blev også bestemt. I et bioraffinaderi er biogasproduktion vigtig for energiudvinding samt for at minimere spild og skabe et ekstra produkt i form af gødning. Energiudnyttelse af PHSR og fermenteringsvæske gennem anaerob nedbrydning svarede til 298 og 285 NmL CH_4 g^{-1} VS_{added}, hhv.

Ved yderligere integration af de forskellige processer i bioraffinaderikonceptet blev en ny biogas-opgraderingsteknologi udviklet. Fremgangsmåde var baseret på CO_2-fiksering af *A. succinogenes* samtidig med produktion af ravsyre og CH_4 af høj renhed. Systemet var i stand til at opnå 95.4% CH_4, hvilket svarer til renhedsgraden som kommercielle biogas-opgraderingteknologier leverer.

Resultater opnået i denne undersøgelse er den første rapport for udnyttelse af makroalger, hamp og jordskokke-knolde som biomasse til fermentativ ravsyreproduktion. Det blev vist, at de tre biomasser er attraktive biomasseråstoffer til ravsyreproduktion, hovedsagelig på grund af deres høje indhold af kulhydrat. Et casestudie af et bioraffinaderikoncept med makroalger beskriver potentialet af PHSR til fremstilling af en lang række yderligere produkter, såsom ω-3 og ω-6 fedtsyrer, biodiesel, protein, foder, biogas og gødning, og kan dermed diversificere bioraffinaderiets produktportefølje.

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1 Introduction

1.1 Climate and energy

In recent times, society has become more aware of the future opportunities offered by the prospect of a sustainable economy, one that is based on renewable resources and energy e.g. wind, geothermal, solar, waste and biomass (Figure 1). Energy generation through utilization of renewable energy sources is rapidly increasing as many governments are taking measures to reduce our civilizations excessive consumption of oil.

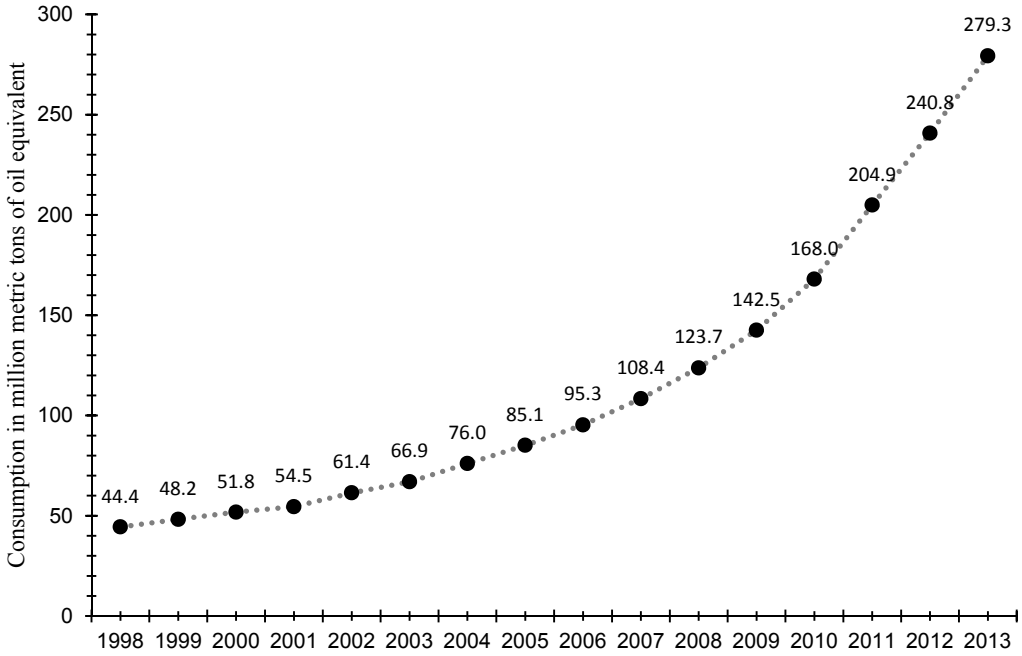

Figure 1. Energy generation from renewable sources during years 1998-2013 including wind, geothermal, solar, biomass and waste, not accounting for cross-border electricity supply. Converted on the basis of thermal equivalence assuming 38 percent conversion efficiency in a modern thermal power station (British Petroleum, 2014).

Within the scientific community there is a general consensus that greenhouse gas emissions (mainly CO_2) arising from the combustion of fossil fuels, material and chemical production, and land-use alteration as a consequence of human activities, are destabilizing the Earth's climate and causing global warming (Hopewell et al., 2009; Loarie et al., 2009; Ramanathan and Feng, 2008).

It has been increasingly recognized that no single solution can relieve the world of its dependency on oil and for that to be realistically possible, collective actions are necessary, including changes in people's behavior, fuel

and vehicle technologies, public transport, power generation and infrastructure (Pickett et al., 2008).

The carbon content of biomass permits combustion or gasification processes in dedicated power plants, where biomass itself serves as a renewable energy source to generate both thermal and electrical energy (Nunes et al., 2014). Though this process can generate power in the form of electricity, it doesn't solve the need of producing biofuels that can be used as fuel for combustion engines found in the vast majority of vehicles used in all forms of transport today.

1.2 Biorefinery

Besides petroleum, biomass is the largest source of carbon-rich material available on Earth, and through sustainable utilization of biomass, a large portion of petroleum based fuels and chemicals can be replaced (Ragauskas et al., 2006). One of the proposed solutions to how that can become reality is to replace petroleum derived products with bio-based products by means of the so-called biorefinery (Kamm et al., 2005). A biorefinery can be described as a facility that integrates biomass conversion processes and technologies in a sustainable and efficient way to produce a variety of marketable products (food, feed, chemicals, and materials) and energy (biofuels, power and/or heat) from biomass (FitzPatrick et al., 2010).

Biomass used as raw material in biorefineries can be categorized into four main categories: Agricultural, forestry, aquatic-, domestic- and industrial organic residues (Cherubini, 2010). Available within these different categories is biomass of vast diversity, however only four constituents found in biomass are of major significance for production of biofuels and industrial products:

- Saccharides
- Lignin
- Triglycerides
- Proteins

Besides these rudimentary structural molecules there is great variety of biomass derived compounds that have additional commercial applications (Kamm et al., 2005). The main common constituents are present in biomass in different amounts, and all of them, except for lignin, can be converted into biofuels such as bioethanol and biogas. Biomass with high carbohydrate

content is however required for bioethanol production through fermentation of sugars, while biogas can be produced from all constituents via anaerobic digestion.

Bioethanol is especially of interest as it can be added in different ratios to gasoline and, if containing <15 % bioethanol, can then used as fuel in most cars having gasoline combustion engines, without requiring making any changes to the engine. Biogas, on the other hand, is of interest due to its various applications and since it can be easily produced from process effluent or process waste coming from e.g. a biorefinery facility, thereby recovering energy. Also, large regions in Europe have extensive infrastructure when it comes to biogas production and utilization.

Production of biochemicals is commonly done through extraction from biomass or via bioconversion processes such as fermentation. The most important biochemicals are the so called platform chemicals (chemical building blocks) (Holladay et al., 2004). If biorefineries are to have a noticeable impact on reducing oil consumption in the future, the biorefinery product portfolio should include platform biochemicals (Fernando et al., 2006). These platform chemicals can be utilized to synthesize a spectrum of other valuable chemicals that can replace their petrochemical derived equivalents (Kamm et al., 2005).

Which biofuels, biochemicals and compounds are produced in a biorefinery is largely dependent on the chemical composition of the specific feedstock as well as which pretreatment, saccharification, conversion and extraction techniques are used (Menon and Rao, 2012; Philp et al., 2013; Sheldon, 2011).

1.3 Objectives and thesis structure

The main objectives of this PhD project were to investigate the bioethanol, biogas and/or succinic acid production potential from high yielding lignocellulosic crops, Jerusalem artichoke (*Helianthus tuberosus* L.) and industrial hemp (*Cannabis sativa* L.) as well as macroalgae biomass (*Laminaria digitata*). Additionally, a considerable part of this PhD project was dedicated for developing a novel technology able to simultaneously upgrade biogas and produce succinic acid. Specific objectives were:

- Characterize the chemical composition of Jerusalem artichoke and industrial hemp, as well as characterization of macroalgae *L. digitata*.

- Evaluate changes in lignocellulosic biomass composition and identify at which harvest time the biomass is best suited for use in a biorefinery.
- Investigate different pretreatment and hydrolysis methods and evaluate their effectiveness.
- Determine if solid residue leftover from enzymatic hydrolysis can be utilized for generating additional products to the biorefinery product portfolio.
- Assess if these biomasses are well suited for production of bioethanol, biogas and/or succinic acid production.
- Test *Actinobacillus succinogenes* 130Z for succinic acid production on Jerusalem artichoke, hemp and macroalgae hydrolysates and evaluate the effects different hydrolysates have on fermentation performance parameters such as production rate, succinic acid yield and concentration.
- Develop a novel technology capable of simultaneously upgrade biogas and produce succinic acid using *A. succinogenes* 130Z, and determine if increasing CO_2 solubility through increasing atmospheric pressure within the system positively affects succinic acid yield and titer as well as methane purity.

In Chaper 2, the chemical composition, production, pretreatment and hydrolysis of the specific lignocellulosic- and brown macroalgae biomasses is covered. Advantages and limitations of using these types of biomasses as feedstock for biorefinery are highlighted.

In Chapter 3, different approaches to bioconversion of Jerusalem artichoke, industrial hemp and *L. digitata* are investigated. Also, what other products can potentially be produced in a biorefinery concept utilizing these biomass feedstocks.

In Chapter 4, a case study of a proposed macroalgae biorefinery concept is presented. The flow of material between processes is shown, and how process integration can minimize waste and CO_2 emissions.

2 Biorefinery feedstocks

Production of biofuels, biochemicals and biomaterials through utilization of food crops such as corn and sugarcane has come under scrutiny in recent years where the sustainability of such processes has been repeatedly questioned (Charles et al., 2007; Naik et al., 2010). Increased competition for land and water; high production cost; extensive use of fertilizers; and limited reduction of net greenhouse gas emissions once land-use change is taken into account are among the main arguments against the production of these so called first generation products (Sims et al., 2010). Therefore there is need to identify and utilize other renewable and more sustainable types of biomass as feedstock for production of bio-based products.

2.1 Lignocellulose

Production of second generation biofuels and bio-based products utilizes lignocellulosic biomass, the most abundant type of land based biomass on Earth. Lignocellulose can be used to produce chemicals that currently are produced through petrochemical route (Gallezot, 2012). Lignocellulose includes virgin biomass (e.g. trees, bushes and grasses), waste biomass from agriculture and forestry (straw, sugarcane bagasse etc.) as well as energy crops (Wu et al., 2014). Lignocellulosic energy crop is high yielding lignocellulosic biomass that generates large quantities of biomass compared to other similar types of feedstocks. Switchgrass and Miscanthus are examples of energy crops that has been widely investigated for biofuel production (Griffith et al., 2014; Heaton et al., 2008), while other promising energy crops such as Jerusalem artichoke (Paper I, II) and industrial hemp (Paper IV, V) have received less attention.

Additionally, natural pest and disease resistance as well as frost and drought tolerance are beneficial traits that some lignocellulosic energy crops e.g. Jerusalem artichoke and hemp exhibit. Risk of failed harvests can thereby be reduced, as sudden undesirable changes in weather won't seriously affect the viability of the biomass.

2.1.1 Chemical composition

Cellulose is the main component of lignocellulosic biomass, while other major constituents are hemicellulose and lignin (Figure 2). Cellulose is a polysaccharide consisting of glucose molecules bound together by β(1-4) bonds. These long polysaccharide chains then form different structures called crystalline and amorphous cellulose (Zaldivar et al., 2001). Like cellulose,

hemicellulose is a polysaccharide, however instead of containing only glucose it consists of a combination of hexoses and pentoses that includes sugars such as xylose, arabinose, mannose, galactose and glucose (Scheller and Ulvskov, 2010). Lignin is a complex hydroxylated and methoxylated phenylpropane polymer that forms covalent bonds with hemicellulose. This complex and robust structure gives structural strength to plant stems and trunks, but also resistance to degradation (Guo et al., 2001).

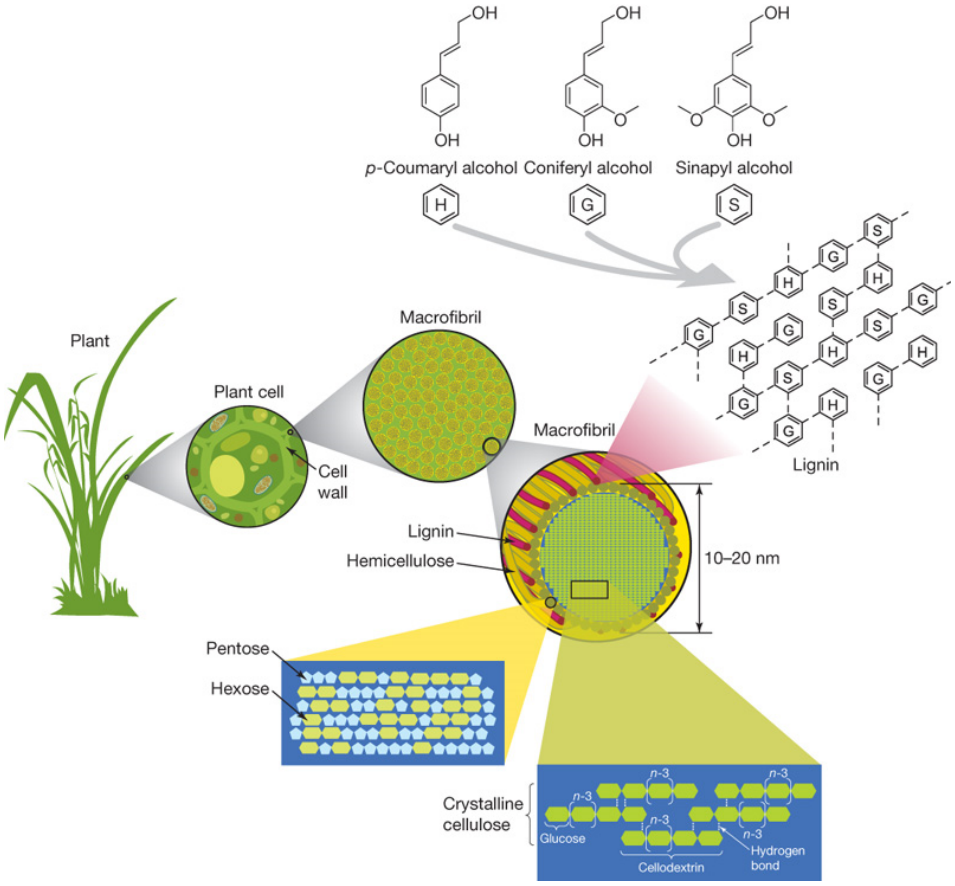

Figure 2. Structure of lignocellulose. Reprinted by permission from Nature Publishing Group: Nature (EM Rubin Nature 454, 841-845 (2008) doi:10.1038/nature07190), copyright 2008.

Content of cellulose, hemicellulose and lignin in lignocellulosic biomasses widely utilized as feedstock for biofuel production or for other biological conversions is commonly 30-50 %, 15-25% and 15-30%, respectively (Carroll and Somerville, 2009).

The chemical composition of industrial hemp presented in Papers IV and V shows that cellulose content of hemp is high (40-46 %) and when combined with 14-19 % hemicellulose the carbohydrate content becomes >55 %. The cellulose content is higher than what is reported for sugarcane bagasse, corn

stover and wheat straw, but lower than that of e.g. Eucalyptus. However, when the total carbohydrate content is compared they are all very similar (Carroll and Somerville, 2009).

Jerusalem artichoke is different from other conventional high yielding energy crops since it produces underground non-lignocellulosic root vegetables (tubers) in addition to the above ground lignocellulosic biomass (Paper I, II; Kosaric et al., 1984). In Paper I the chemical composition of both lignocellulosic- and tuber biomass is presented. Cellulose content of the lignocellulosic Jerusalem artichoke biomass was reported to be rather low (15-24 %). Harvest time was however shown to have great influence on the content of cellulose, where harvesting the biomass later, e.g. in December generally resulted in higher cellulose content. Moreover, hemicellulose (11-14 %) and lignin content (14-21 %) was not influenced to the same extent by harvest time as cellulose was (Paper I). The underground tubers are however rich in inulin, amounting to 10–20% of fresh tuber weight. Inulin is a linear polysaccharide consisting of fructose bonded by β(2→1) linkages that are terminated by a glucose molecule bonded to fructose by a α(2→1) bond (Barclay and Ginic-Markovic, 2010). Jerusalem artichoke tubers have potential for numerous uses including animal feed, production of purified inulin for use as dietary fiber, high fructose syrup or production of bioethanol or other biochemicals through bioconversion (Paper I, II; Li et al., 2013).

2.1.2 Influence of clone selection and harvest time on biomass productivity and composition

Biomass yields for lignocellulosic biomasses (corn stover, sugarcane bagasse and wheat straw) are in the range of 4-11 t ha^{-1} dry matter (Del Río et al., 2012; Kadam and McMillan, 2003; Sakdaronnarong and Jonglertjunya, 2012). However, energy crops such as Switchgrass and Miscanthus are reported to produce around 20-30 t ha^{-1} of dry matter (Lewandowski and Heinz, 2003).

Identifying specific clones or genotypes of certain types of energy crops which are better at coping with less favorable climates e.g. in northern Europe, or generate higher biomass yields is of high importance. Studies have shown that the biomass productivity as well as biomass composition can largely vary when comparing numerous clones or genotypes (Adler et al., 2006; Berdahl et al., 2005; Clifton-Brown and Lewandowski, 2002; Vogel and Mitchell, 2008). Additionally, many of the same studies also investigate how seasonal time of harvest affects yield and the chemical composition. Few

studies have been conducted where the aim was to determine at what time of year is best to harvest lignocellulosic energy crops (Christian et al., 2008; Kreuger et al., 2011; Lewandowski et al., 2003; Matías et al., 2013). The ideal biomass feedstock for a biorefinery producing its main products through biological conversions should preferably generate high biomass yields per area, and exhibit high carbohydrate content.

Studies have been conducted where comparison is made between different clones or genotypes of non-conventional energy crops such as industrial hemp or Jerusalem artichoke as well as comparing the effect of harvesting times (Paper I; Amaducci et al., 2008; Gunnarson et al., 1985; Kosaric et al., 1984; Kreuger et al., 2011). Even when cultivated in the cold climate in Northern Europe industrial hemp has been reported to yield as much as 16 t ha^{-1} of dry biomass (Amaducci et al., 2008) which is significantly higher than that of corn stover, sugarcane bagasse and wheat straw. In Paper I where eleven clones where cultivated and harvested at three occasions, it was reported that the highest yield of Jerusalem artichoke lignocellulosic biomass (35 t ha^{-1} dry matter) was up to three times higher than other common types of lignocellulosic biomass. These biomass yields were still comparable to that of Switchgrass and Miscanthus.

Furthermore, as reported in Paper I, in addition to the Jerusalem artichoke lignocellulosic biomass, the plant also produced considerable amounts tubers. It was observed that late harvesting, when the lignocellulosic biomass had the highest cellulose and hemicellulose content, also resulted in high yield of inulin from tubers (around 6 t ha^{-1} dry matter).

2.1.3 Pretreatment

Pretreatment step is necessary to increase the porosity of lignocellulosic biomass structure, so that hydrolytic enzymes can get improved access to the cellulose fibres (Wyman et al., 2005). The increased porosity is mainly due to lignin removal or hemicellulose hydrolysis, but to what extent is greatly dependant on which pretreatment method is used (Alvira et al., 2010). Pretreatment methods can be categorized as being physical, chemical, physicochemical or biological depending on the nature of the pretreatment. Harsh conditions involving addition of acid or alkaline, oxidative agents, high temperatures and/or pressure are often used in combination (Alvira et al., 2010). Pretreatment methods such as steam explosion (physicochemical) or chemical pretreatments using dilute (0.5-2%) H_2SO_4 or NaOH in combination with high temperature (120-220 °C) are the most common

methods (Taherzadeh and Karimi, 2008). The use of H_2O_2 or other oxidative agents has been shown to be effective at high pH, commonly pH 10-12 for effective removal of lignin (Hendriks and Zeeman, 2009).

Removal of lignin and/or hemicellulose prior to hydrolysis using pretreatment is directly linked with improving the enzymatic digestibility of cellulose fibres (Ohgren et al., 2007; Saengkanuk et al., 2011). Solubilization of hemicelluloses through thermal pretreatment can however result in formation of inhibitory compounds which can inhibit enzymatic hydrolysis and bioconversion processes (Klinke et al., 2004; Liu, 2006). The main inhibitory compounds being; sugar degradation products such as furfural and hydroxymethylfurfural, acetic acid released from hemicellulose, as well as aromatic and phenolic compounds as result of lignin degradation (Palmqvist and Hahn-Hägerdal, 2000). Thus, assessment of which inhibitory compounds are formed during pretreatment is important in order to minimize inhibition during bioconversion. Utilization of novel types of feedstock requires an extensive assessment of which pretreatment to use, since pretreatment conditions can vary greatly depending on the nature and composition of the biomass.

Many pretreatments catalyse hemicellulose hydrolysis mainly into xylose, a pentose (C5) sugar which many wild-type microorganisms are unable to ferment (Lloyd and Wyman, 2005).

In Papers IV and V, the performance of different pretreatment methods were tested on hemp biomass. Dilute H_2SO_4 pretreatment was exclusively used in Paper V, where the effect of varying the concentration of acid (0 – 2 %) as well as pretreatment temperature (140, 180 °C) on biomass composition was evaluated. However, in Paper IV the effectiveness of different diluted chemical agents, H_2SO_4, NaOH and H_2O_2 was tested, and if their use affected biomass composition, saccharification and fermentation processes differently.

2.1.4 Saccharification

Following appropriate pretreatment, accessibility for cellulolytic enzymes to the cellulose fibers is greatly increased (Wyman et al., 2005). Enzyme mixtures usually containing cellulases and β-glucosidase are used to hydrolyze cellulose to glucose. Cellulases (endo-β-1,4-glucanglucanhydrolases, exo-β-1,4-glucancellobiohydrolases) catalyze the hydrolysis of cellulose to cellobiose (glucose dimer), while β-glucosidase subsequently hydrolyzes cellobiose into monomeric glucose (Parisi, 1989). The efficiency of cellulolytic enzymes has been shown to be affected by

product inhibition (feedback inhibition) when cellobiose and glucose concentrations rise during enzymatic hydrolysis of cellulose (Palmqvist and Hahn-Hägerdal, 2000). After enzymatic hydrolysis, glucose as well as other monosaccharides such as xylose are present in the hydrolysate and available to microorganisms for fermentation.

As presented in Paper IV and V, pretreatment greatly influences the efficiency of enzymatic hydrolysis of cellulose fibres in pretreated hemp material. In paper V, an increase of around 69% in glucose release was observed when hemp material was pretreatment with 1% H_2SO_4 opposed to not adding acid at 180 °C. Furthermore, when using the most favourable pretreatment conditions, glucose yield after enzymatic hydrolysis reached 74.3% were reported. Similar trends have been reported in studies investigating the effect of pretreatment on enzymatic hydrolysis using other types of biomasses (Ferreira et al., 2011; Xu et al., 2011).

In Paper IV, results from enzymatic hydrolysis of hemp material pretreated with three different diluted chemical agents (0-3% H_2SO_4, NaOH or H_2O_2) were reported (Figure 3). Results showed that the best pretreatment conditions used in Paper V (1% H_2SO_4 at 180°C) led to the lowest glucose concentration and yield during enzymatic hydrolysis (Figure 3A), compared the other chemical agents. However, when using this method, the second highest overall sugar yield (glucose + xylose) of 70.4% after pretreatment and hydrolysis was achieved. The highest glucose concentration (31.3 g L^{-1}) and yield (90.0%) was observed after hydrolysis of hemp material pretreated with 3% H_2O_2 at 121°C (Figure 3C). Furthermore, this method resulted in the highest overall sugar yield (glucose + xylose) of 73.5% after pretreatment and hydrolysis.

While the use of enzymes for hydrolysis of cellulose is standard practice for effective saccharification of pretreated lignocellulosic material, certain biomasses require neither pretreatment nor addition of enzymes for effective hydrolysis of polysaccharides present in the biomass. Inulin present in Jerusalem artichoke tubers can for example be hydrolyzed through dilute H_2SO_4 hydrolysis, where very low acid concentrations are sufficient for efficient inulin hydrolysis to take place (Paper II; Pekić et al., 1985). In Paper II tuber inulin was hydrolysed at 100 °C using only 0.2 % H_2SO_4 solution, where no pretreatment was used and no hydrolytic enzymes were added.

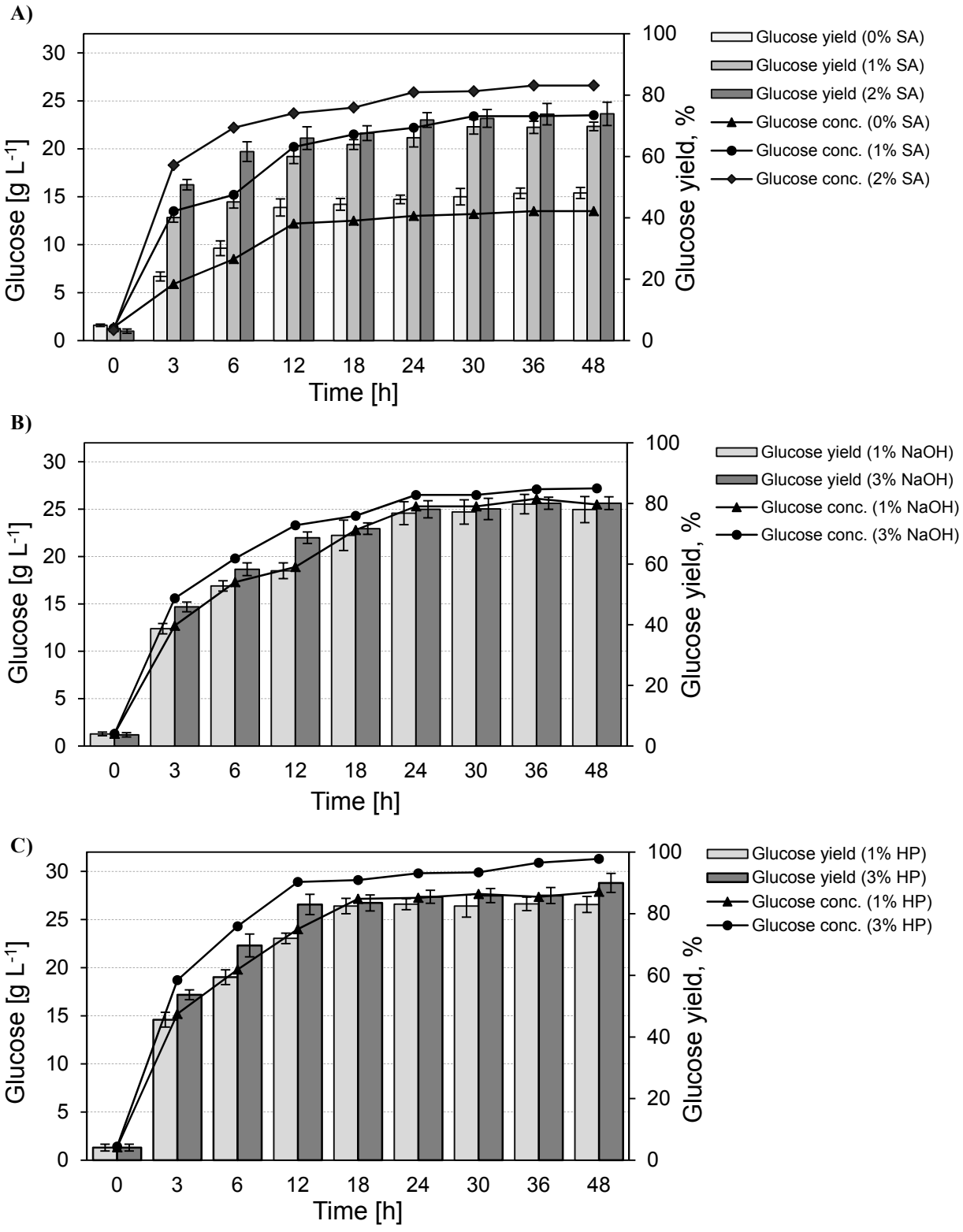

Figure 3. The course of enzymatic hydrolysis (A - hemp pretreated with H_2SO_4; B - hemp pretreated with NaOH; C – hemp pretreated with H_2O_2; conc. – concentration; HP – hydrogen peroxide; SA – sulfuric acid; glucose yield – error bars represent standard deviation).

2.2 Macroalgae

While utilization of lignocellulosic biomass as feedstock in biorefineries is expected to greatly increase in the near future, problems related to land space, water resources and fertilizer use will inevitably occur as the human population grows to almost 10 billion by year 2050 (Ajanovic, 2011; Enquist-Newman et al., 2014). Therefore, while continuing research efforts on the different applications of lignocellulosic biomass, finding more efficient and sustainable sources of biomass will be essential. In this context, aquatic biomasses such as macroalgae have been suggested as potential candidates to be used in future biorefinery concepts for the production of third generation transportation fuel, energy, chemicals and materials (Enquist-Newman et al., 2014). Macroalgae encompass a number of attributes of a model feedstock that can help with meeting the challenges involved with the steadily increasing demand for energy and food. Since macroalgae does not required land, neither fresh water nor fertilizer for growing, cultivation of this type of biomass avoids having antagonistic impacts on food production and resource availability (Enquist-Newman et al., 2014; Jung et al., 2013).

Macroalgae biomass growth and chemical composition are considerably affected by their environmental conditions where temperature, light, nutrient availability, salinity and water currents are the main factors. To what extent the macroalgae is affected by the different environmental factors is highly dependent on their taxonomical classes and species (Holdt and Kraan, 2011; Jung et al., 2013).

The brown algae *Laminaria digitata* is one the most promising macroalgae species for utilization as biorefinery feedstock (Paper VI; Jung et al., 2013).

2.2.1 Composition

Brown macroalgae species such as *L.digitata*, are of particular interest since they often contain high carbohydrate and protein content, while they do not contain any lignin (Holdt and Kraan, 2011). The absence of lignin means harsh pretreatments are at times unnecessary prior to enzymatic saccharification of polysaccharides. Sugars can therefore be extracted more readily than compared to land based biomass such as lignocellulose (Paper VI; Enquist-Newman et al., 2014; Wargacki et al., 2012).

From species with high protein content, protein meal for animal feed can be produced to add value to the biorefinery (Holdt and Kraan, 2011). However, the protein content of macroalgae varies greatly between species. Brown

macroalgae such as *L. digitata* generally contain 3-15 % of dry weight is protein, which is low compared to green macroalgae (Fleurence, 1999).

Lipid content of macroalgae is in general considered to be low compared to other types of biomass feedstocks (Mabeau and Fleurence, 1993) and *L. digitata* is reported to be one of the species containing the least amount (0.3-3 % dry weight) of lipids (Fleurence et al., 1994; Holdt and Kraan, 2011). While the lipid content is considered to be low in macroalgae, it has been reported that a considerably large fraction of the lipid components are essential polyunsaturated fatty acids (PUFA) such as ω-3 and ω-6 fatty acids (MacArtain et al., 2007). The protein and lipid content of the *L. digitata* biomass utilized in Paper VI was found to be 3.5 % and 0.8 %, respectively. These two constituents were therefore both on the lower side of values reported for *L. digitata*.

Carbohydrate content of dry *L. digitata* and other brown macroalgae species can reach up to 60 % or even higher (Paper VI; Holdt and Kraan, 2011; Wei et al., 2013). The carbohydrates present in *L. digitata* are mainly laminarin and mannitol, while some cellulose and alginate is also present. Like cellulose, laminarin is completely composed of glucose molecules. However, unlike cellulose, the glucose units in laminarin are linked together by β(1→3) bonds forming a linear polysaccharide with branches composed of glucose molecules with β(1→6) bonds (Adams et al., 2014). Mannitol, an alcohol form of the sugar mannose, is present in *L. digitata* in free monomeric form and is easily extracted. Both glucose and mannitol can be fermented by numerous microorganisms, while a very limited number is able to metabolize uronic acids, the constituents of alginate (Paper VI; Enquist-Newman et al., 2014; Wargacki et al., 2012). Since the content of carbohydrates in the algae biomass can vary greatly between seasons, even between months or weeks (J M M Adams et al., 2011), the content of fermentable sugars is of high importance for a biorefinery producing its main products through fermentation.

In Paper VI was reported that the total carbohydrate content of the *L. digitata* biomass harvested in August was 77.6 % w w^{-1} of dry biomass, where glucose and mannitol content was measured to be 69.6 % and 8.0 % w w^{-1} of dry biomass, respectively. While the reported carbohydrate content was higher than reported in other studies investigating *L. digitata* (J M M Adams et al., 2011; Holdt and Kraan, 2011) the results can only be explained by favorable environmental conditions leading up to the harvesting.

2.2.2 Saccharification

Due to the absence of lignin and the porous structure of the biomass, macroalgae is much less ridged than lignocellulose. Therefore polysaccharides found in macroalgae are vulnerable to hydrolysis. Some studies report using dilute acid or alkaline pretreatment prior to hydrolysis, while other studies elect not to use pretreatment (John et al., 2011; Tan and Lee, 2014; Trivedi et al., 2013). Which methodology is used largely depends on which macroalgae species was utilized, carbohydrate content and their degradability. Most common methods of hydrolyzing macroalgae polysaccharides are direct dilute acid hydrolysis or enzymatic hydrolysis (Borines et al., 2013).

Numerous investigations have been conducted where hydrolysis of macroalgae biomass is done prior to some sort of fermentation, commonly bioethanol fermentation (Borines et al., 2013; Jang et al., 2012; Tan and Lee, 2014; Trivedi et al., 2013). However, a common problem in many of these studies is that the concentrations of fermentable sugars present in hydrolysate after either acid and/or enzymatic hydrolysis is low. To show that macroalgae can realistically replace other less sustainable biomasses as feedstock for biorefinery bioconversion processes the concentrations of fermentable sugars need to at least exceed 100 g L^{-1}, preferably much more.

In paper VI very high solid loading (up to 250 g L^{-1}) of dried *L. digitata* was used for enzymatic hydrolysis without any previous pretreatment step. Results presented in Paper VI show that even when using such high biomass solid loading (250 g L^{-1}) of *L. digitata*, enzymatic hydrolysis efficiency of 78% was reached where the final concentrations of glucose and mannitol in the hydrolysate corresponded to 119.4 g L^{-1} and 18.8 g L^{-1}, respectively (Figure 4). Reaching these high sugar concentrations is critical if bioconversion routs following the hydrolysis step are to produce sufficient quantity of product so that later downstream processing steps can become economically feasible.

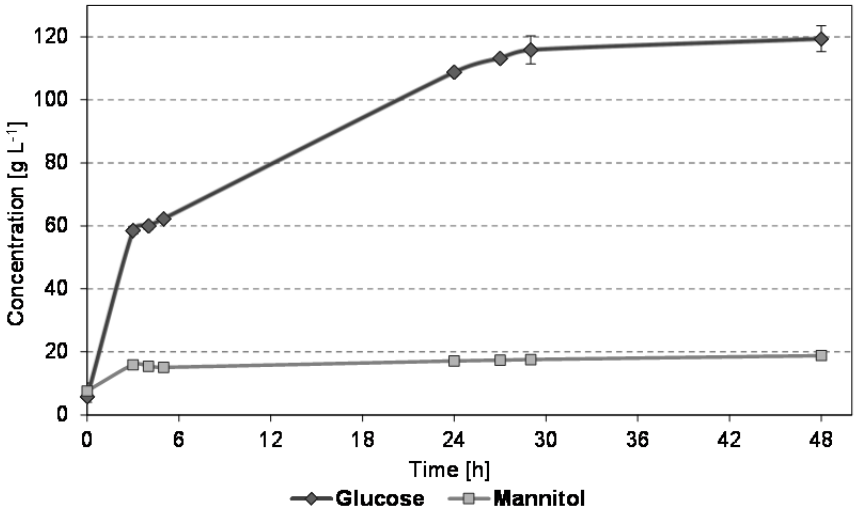

Figure 4. Enzymatic hydrolysis of dried *L. digitata* material.

Moreover, in Paper VI the *L. digitata* post hydrolysis solid residue (PHSR) was collected and analyzed. Results showed that by removing most of the laminarin and mannitol from the biomass other biomass constituents were up-concentrated. It was found that protein content of PHSR was 3.5 times higher (from 3.45 to 12.15 % dry matter) than what was measured in the original dried *L. digitata* material. The same trend was observed with lipid content, which increased even more, or 8.6 times (from 0.77 to 6.61 % dry matter). The up-concentration of these constituents definitely adds value and prospect of further utilization of PHSR for producing other products within the biorefinery concept.

3 Bioconversions

3.1 Bioethanol

As a result of increased public interest, scientific research concerning the production of biofuels has increased dramatically. Nevertheless, the majority of current production processes for biofuels such as bioethanol, are single production chains. Additionally, these processes usually require feedstock e.g. sugar from sugarcane or corn starch, which results in competition with food and feed production. A great escalation in exploitation of food crops such as sugarcane or corn for biofuel production is thereby limited (FitzPatrick et al., 2010). Bioethanol produced from these types of biomass feedstocks are generally referred to as first generation bioethanol. Yeasts are the most common microorganisms used for production of bioethanol, where *Saccharomyces cerevisiae* is the most widely used species. *S. cerevisiae* is known to ferment very efficiently sugars such as glucose, fructose and sucrose into ethanol and CO_2. Theoretically, 2 moles of ethanol can be obtained from fermentation of 1 mole of hexose (C6) sugar such as glucose (Eq. 1).

$$C_6H_{12}O_6 \rightarrow 2\ C_2H_5OH + 2\ CO_2 \qquad \text{Eq. 1}$$

Ethanol produced through fermentation of lignocellulosic biomass is commonly referred to as second generation bioethanol. The production process generally consists of the following steps: Size reduction, pretreatment, enzymatic hydrolysis, fermentation and ethanol recovery (Carroll and Somerville, 2009).

The production of bioethanol from lignocellulosic raw material has been widely investigated in recent decades (Suurs and Hekkert, 2009). During that time, numerous plant biomass feedstocks with high cellulose content (30-45% of dry matter) such as wheat straw, rice straw, corn stover and sugarcane bagasse have been studied and tested as feedstocks for ethanol fermentation (Carriquiry et al., 2011; Carroll and Somerville, 2009).

Full commercialisation of second generation bioethanol appears to remain some years away, even though research and development has been ongoing for several decades and large investments have been made in pilot- and demonstration scale plants in US, Europe and elsewhere (Sims et al., 2010). Though second generation bioethanol is still not able to compete cost-effectively with first generation bioethanol or fossil derived transportations

fuels there have still been made significant improvements to the process (Geddes et al., 2011).

It has been shown that *S. cerevisiae* can normally ferment glucose present in lignocellulose hydrolysates, however its inability to ferment xylose has been a major obstacle for second generation bioethanol production (Jeffries, 2006; Zhang and Geng, 2012). Efficient xylose fermentation is essential for second generation bioethanol to become economically viable (Zhang and Geng, 2012), whether that would be fermentation into ethanol, or possibly production of other biochemicals through fermentation of xylose in a separate process using the biorefinery concept (Kaparaju et al., 2009).

Fermentation of different hemp hydrolysates using *S. cerevisiae* was conducted in Paper V, where ethanol yield was reported 74-92% of theoretical yield, though the highest ethanol concentration reached was 10.0 g L^{-1}, which is low. The low concentrations of ethanol are mainly explained by the low concentrations of glucose during fermentation, as availability of sugar is the main factor determining the final concentration of ethanol. Though few investigations have been made on bioethanol production from hemp some have reported ethanol concentrations >20 g L^{-1} (Sipos et al., 2010). In Paper V some common bottlenecks in second generation bioethanol production are evident e.g. in pretreatment step where solid loading is 10% w v^{-1}, which results in excessive water usage, and since yeast is unable to ferment xylose (main sugar present in the liquid fraction) large amounts of wastewater are generated. Furthermore, during enzymatic hydrolysis of pretreated hemp material 5% w v^{-1} solid loading was used as it has been shown that low yields, process inefficiency and enzyme inhibition occur when solid loading is increased (Kristensen et al., 2009), however the trade-off is low ethanol concentrations after fermentation.

One way to relatively easily increase the availability of fermentable sugars in bioethanol fermentation processes could be to utilize additionally biomass such as Jerusalem artichoke tubers, which require little acid and no enzymes for hydrolysis of polysaccharides. As discussed in Paper I, the bioethanol production potential of Jerusalem artichoke tubers has been reported between and 3.060 and 11.000 L ha^{-1} which is in the same range as, or even more than, from sugarcane and corn.

The economic side of operating a biorefinery cannot be overlooked, and since second generation bioethanol is currently not able to compete price wise with fossil fuels, due to process operation costs, fermenting the carbohydrates into

a biochemical, of more added value, should be considered. Instead of bioethanol, another biofuel e.g. biogas could be produced from process residues and effluents.

3.2 Biogas

Biogas is another renewable energy carrier which can be used as vehicle fuel, for heat and power generation or injected into the national gas grid after purification (upgrading) into biomethane (Holm-Nielsen et al., 2009). Biogas is produced through anaerobic digestion, during which microorganisms catalyse a collection of biological processes in order to break down biodegradable material in organic wastes, residues and crops (Gujer and Zehnder, 1983). The composition of biogas can vary depending on several factors e.g. substrate and stability of the process, however, normally it is a mixture containing 50-75% CH_4 and 25-50% CO_2 (Ryckebosch et al., 2011). It is though that at least 25% of all bioenergy generated in the future within the European Union can originate from biogas, produced from wet organic materials (Holm-Nielsen et al., 2009).

As mentioned in Paper I biogas production from the lignocellulosic part of Jerusalem artichoke has been shown to be a good feedstock for biogas production through anaerobic digestion with biogas yields up to 590 NmL g^{-1} VS. Tubers have also been used for biogas production, however due to their high content of carbohydrates, other kinds of fermentations such as ethanol fermentation are preferred (Szambelan et al., 2004). Also, as pointed out in Paper V one way to recover additional energy from bioethanol production is to use stillage as feedstock for anaerobic digestion.

In Paper VI the biochemical methane potential (BMP) of macroalgae *L. digitata*, post hydrolysis solid residue (PHSR) and fermentation broth was determined and compared (Figure 5). From dried *L. digitata* 289 NmL CH_4 g^{-1} VS were produced, while the results also showed that considerable amount of energy, 298 and 285 NmL CH_4 g^{-1} VS, could be recovered from PHSR and fermentation broth, respectively. The BMP results for these different feedstocks were significantly higher than the 184 – 238 NmL CH_4 g^{-1} VS reported in other studies investigating the BMP of *L. digitata* biomass (J. M M Adams et al., 2011; Vanegas and Bartlett, 2012). This is most likely due to the unusually high content of the readily degradable polysaccharide laminarin (69.6%) as well as mannitol (8.0%) in the dried macroalgae material. Methane yield observed from fermentation broth in Paper VI (285 NmL CH_4 g^{-1} VS) is 12-41% lower than methane yields reported for

comparable feedstocks such as stillage from wheat straw bioethanol production (Kaparaju et al., 2009). Observations made in Paper VI show nevertheless that albeit after removing majority of the carbohydrates from *L. digitata*, PHSR is still a good feedstock for biogas production. The fermentation broth, after succinic acid fermentation, is a waste stream also able to contribute considerably to the energy output of the process, even though carbohydrates were fermented prior to anaerobic digestion.

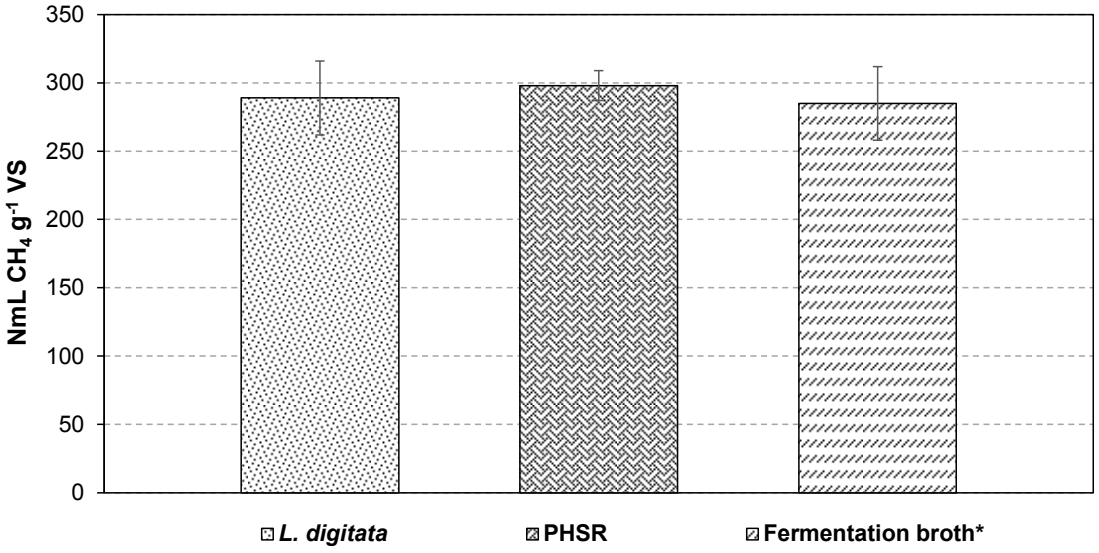

Figure 5. Biochemical methane potential of *L. digitata*, PHSR and fermentation broth. *Methane production as result of anaerobic digestion of fermentation broth with production from succinic acid subtracted.

Cultivating and harvesting biomass solely for biogas production is not a viable option due to the low added value of biogas. However, anaerobic digestion of biorefinery process effluents can be used to recover energy in the form of biogas.

3.3 Succinic acid

Succinic acid is a four carbon diacid with the chemical formula $C_4H_6O_4$ and is a precursor in the chemical synthesis of numerous commodities in agricultural, food, chemical and pharmaceutical industries (Figure 6) e.g. solvents and biodegradable polymers (Cukalovic and Stevens, 2008; Zeikus et al., 1999). Succinic acid can be produced biologically through fermentation by using bacteria such as *Actinobacillus succinogenes* (Guettler et al., 1999), and has been recognized as one of the twelve most promising building block chemicals that can be produced through biological conversion of sugars derived from biomass (Bozell and Petersen, 2010; Fernando et al., 2006).

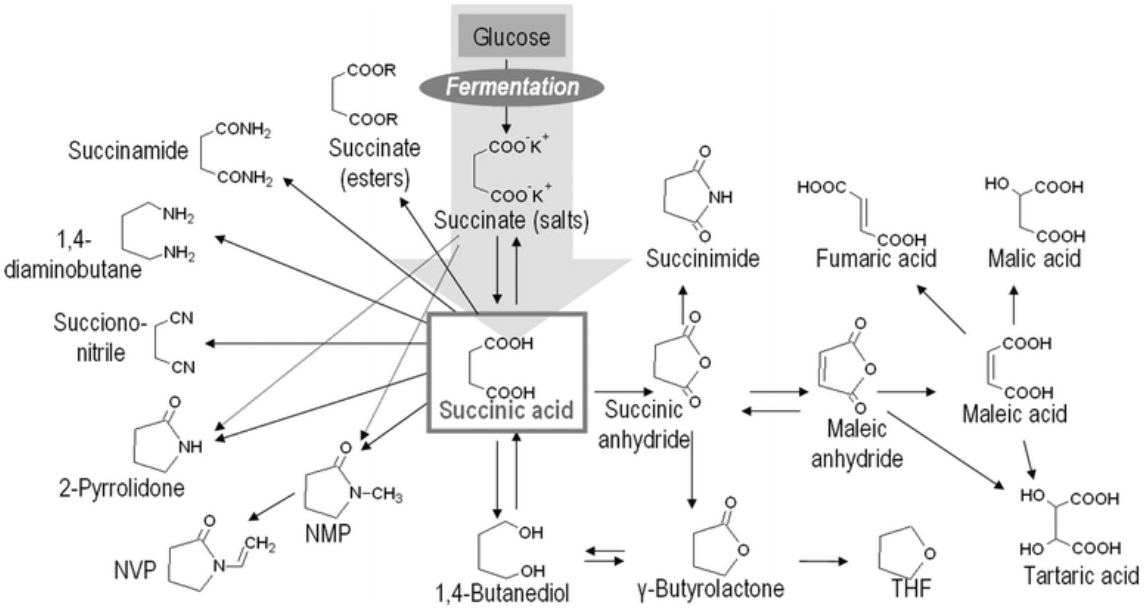

Figure 6. Succinic acid derivatives (Kamm and Kamm, 2007).

A. succinogenes is a facultative anaerobe with high tolerance to initial sugar concentrations, but also tolerates high levels of succinic acid which makes it one of the most promising producers of biosuccinic acid (Guettler et al., 1999; Lin et al., 2008). This bacterium produces succinic acid due to the lack of a complete tricarboxylic acid cycle (Figure 7) as well as a glyoxylate pathway. Furthermore, *A. succinogenes* is able to transport and degrade about twenty different carbohydrates, while also fixating large amounts of CO_2 during fermentation (McKinlay et al., 2010). Hence, being able to metabolize this variety of substrates, the source of feedstock can be flexible and not only contain sugars readily fermentable by other microorgansisms.

During fermentation of sugars e.g. glucose, *A. succinogenes* consumes 1 mol of CO_2 per 1 mol of succinic acid produced (Song and Lee, 2006). In the central metabolic pathways of *A. succinogenes* (Figure 7), the enzyme PEP carboxykinase (PEPCK), which catalyses the conversion of PEP to OAA, can be highlighted as the main reason for CO_2 fixation during fermentation. However, the reverse flux from Pyr to Mal and possibly OAA has also been shown to consume CO_2. It has been shown that the metabolic flux towards succinic acid is highly interrelated with the availability of CO_2. Higher CO_2 availability therefore normally results in higher succinic acid production and less production of by-products (formic acid, acetic acid and ethanol), making CO_2 a key factor in the production of succinic acid by *A. succinogenes* (McKinlay et al., 2010; McKinlay and Vieille, 2008).

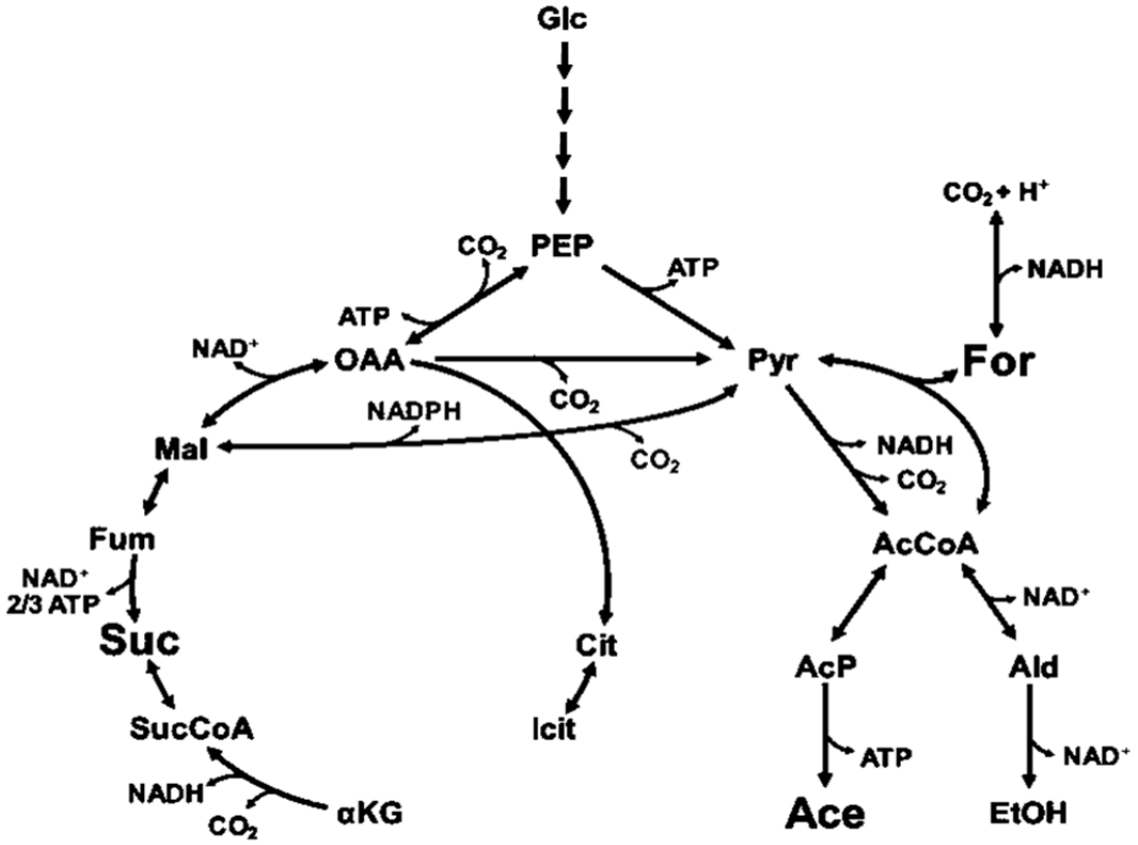

Figure 7. Simplified version of the central metabolic pathways known to be active during *A. succinogenes* mixed acid fermentation. Metabolites: AcCoA, acetyl-CoA; Ace, acetate; AcP, acetyl-phosphate; Ald, acetaldehyde; Cit, citrate; EtOH, ethanol; For, formate; Fum, fumarate; Glc, glucose; Icit, Isocitrate; αKG, α-ketoglutarate; Mal, malate; OAA, oxaloacetate; PEP, phosphoenolpyruvate; Pyr, pyruvate; Suc, succinate; SucCoA, succinyl-CoA.

Substantial CO_2 emission savings in the range of 4.5-5 tonne per tonne biosuccinic acid produced have been estimated compared to emissions of petrochemical derived succinic acid, which can considerably improve the sustainability indicators of biorefineries (Clark et al., 2006; Hermann et al., 2007).

Studies have shown that succinic acid can readily be produced from hydrolysates of different types of lignocellulosic biomass e.g. rapeseed meal, corn stover, corn-, rice- and wheat straw (Chen et al., 2011; Zheng et al., 2010, 2009). One of the studies reported that *A. succinogenes* produced 45.5 g L^{-1} of succinic acid during batch fermentation of corn straw hydrolysate containing mainly a mixture of glucose and xylose, with a succinic acid yield of 80.7% (Zheng et al., 2009).

Paper II was the first study that investigated using Jerusalem artichoke tubers as feedstock for fermentative succinic acid production. In Paper II it was reported that fermentation of tuber hydrolysate mixed at 1:1 ratio with medium resulted in 26.9 g L^{-1} of succinic acid produced at a yield of 74% from a mixture of fructose and glucose present in the hydrolysate. To investigate if the cost of the fermentation process could possibly be lowered, fermentation of undiluted tuber hydrolysate was investigated, which meant not adding any growth medium containing nutrients. Through this approach initial sugars concentrations could be doubled, resulting in succinic acid production of 47.4 g L^{-1}, but at a slightly lower yield, 67%. The observations presented in Paper II showed clearly that succinic acid fermentation of tuber hydrolysate could be carried out without nutrient addition. Though this approach resulted in increased succinic acid production, lower yield and significantly slower bacterial growth was observed, most likely due to reduced availability of nitrogen (Chen et al., 2011) and higher substrate concentrations (Lin et al., 2008).

In paper IV, various mixing rations (0:100, 25:75, 50:50 and 75:25) of medium and hemp hydrolysate were tested in 200 mL batch bottles to investigate if hydrolysate strength affected fermentation performance. Hemp hydrolysates generated after 1% H_2SO_4 or 3% H_2O_2 pretreatment followed by enzymatic hydrolysis were the most promising (highest overall sugar yields), and were selected for succinic acid fermentation. In batch bottles, the highest succinic acid titer, 19.0 g L^{-1}, was reached after fermentation of hemp hydrolysate generated after 3% H_2O_2 pretreatment followed by enzymatic hydrolysis, using 25:75 mixing ratio. In case of both types of hydrolysates, 25:75 mixing ratio resulted in the highest succinic acid titer, while succinic acid yields >78% were only obtained at 75:25 mixing ratio. After conducting fermentations in batch bottles, two mixing ratios 50:50 and 25:75 (medium:hydrolysate) were selected for further investigation. Fermentation of hemp hydrolysates (1% H_2SO_4 or 3% H_2O_2 pretreatment followed by enzymatic hydrolysis) was done using the previously mentioned mixing ratios in 3-L fermenters. The highest succinic acid titer (21.9 g L^{-1}) was observed after fermentation of hydrolysate using 3% H_2O_2 pretreatment and 25:75 mixing ratio. Succinic acid yields increased between 3-9% when fermentations were conducted in fermenters compared to batch bottles, while the highest yields were 82-83% after fermentation of hydrolysate using 3% H_2O_2 pretreatment.

Both Jerusalem artichoke tubers and industrial hemp were both shown to be good feedstocks for succinic acid production. Prior to Papers II and IV neither Jerusalem artichoke tubers nor hemp had been evaluated as feedstock for fermentative succinic acid production.

Paper VI was the first study conducted where macroalgae was utilized as feedstock in a biorefinery concept where succinic acid was the main biorefinery product. Macroalgae species such as *L. digitata*, which contain high amounts of carbohydrates are especially interesting since the biomass needs less pretreatment, and in the case of *L. digitata* the laminarin is readily hydrolysed. In Paper VI the succinic acid production from macroalgae hydrolysate was investigated in batch mode on different scales (200ml batch bottles as well as in 3-L fermenter). When the macroalgae hydrolysate was diluted at a ratio of 1:5 with medium 24.4 g L^{-1} succinic acid were produced at a yield of 86.5% which is one of the highest values ever reported in literature for this bacterium (Beauprez et al., 2010; Borges and Pereira, 2011; Zheng et al., 2009). When conducting the fermentation in fermenter the hydrolysate was diluted at a ratio of 1:1.5 with growth medium and the succinic acid production increased to 33.8 g L^{-1}, but only at yield of 63.2%. The cause for this large change in succinic acid yield between experiments is not obvious. It should however be remarked that the initial substrate concentration was higher in the fermenter experiments than in batch bottles. That could possibly explain the differences in process performance, as different substrate concentrations might well lead to differences in the metabolic fluxes.

3.3.1 Simultaneous biogas upgrading and succinic acid production

Biogas, produced through anaerobic digestion of organic material, is generally composed of 50-75% CH_4 and 25-50% CO_2. Depending on the final use, different processing steps are necessary. For instance, where it's important to have high energy content in the gas, as in vehicle fuel or for grid injection, the CH_4 needs to be purified via a process called biogas upgrading (Ryckebosch et al., 2011). By removing CO_2 from the biogas in the upgrading process, the energy content of the gas increases. Current upgrading technologies such as absorption make use of the physical/chemical properties of CO_2 where it is captured from the biogas (thereby increasing the CH_4 purity) and then released into the atmosphere, contributing to the global warming effect (Ryckebosch et al., 2011). Current upgrading technologies can effectively captures CO_2 from biogas, however none of them have the

ability to convert the CO_2 into another added value commodity. Such a technology could potentially be a revelation for biogas upgrading, since the benefits of integrating two processes in such a way could potentially overcome the cost of CO_2 capture and storage and reduce the cost of biogas upgrading.

In Paper III a novel biogas upgrading technology is demonstrated which makes use of the CO_2 fixation abilities of *A. succinogenes* to simultaneously produce high purity CH_4 and succinic acid. An experimental setup (Figure 8) was built which mainly consisted of a 3-L fermenter containing medium inoculated with *A. succinogenes,* and a gas bag containing biogas (60% CH_4, 40% CO_2). As shown in Figure 8, biogas is recirculated through the system during fermentation, allowing changes in gas composition to be observed. Under anaerobic conditions, the biogas was injected at the bottom of the fermenter. As the biogas bubbles travel through the vessel some of the gaseous CO_2 gets solubilized in the culture broth. As fermentation progresses, CO_2 levels decrease in the liquid phase, due to CO_2 fixation, which according to Henry's law forces more CO_2 in the gas phase to be dissolved into the culture broth. In this way the CH_4 content of the biogas is increased.

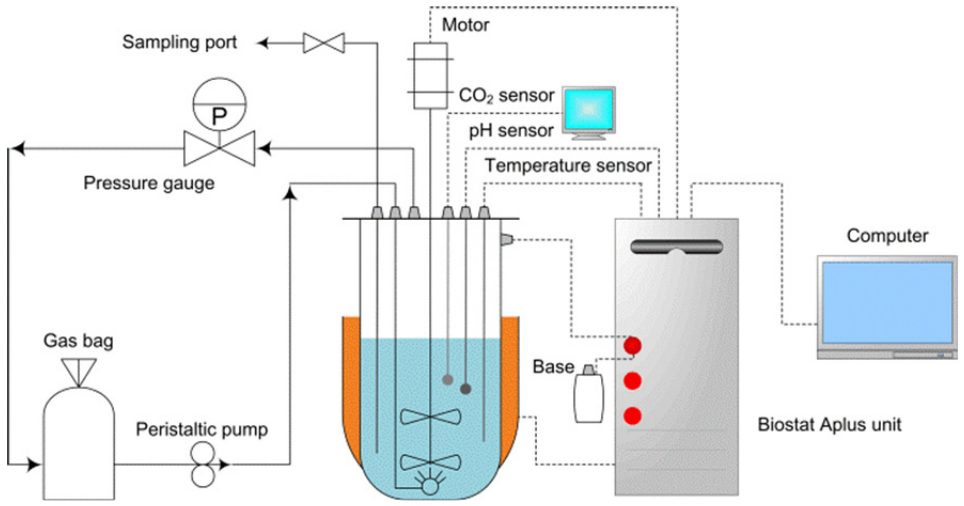

Figure 8. System setup for simultaneous biogas upgrading and succinic acid production.

Varying the gas-liquid ratio and CO_2 partial pressure resulted in changes in process performance (Table 1). At both gas-liquid ratios, increasing the CO_2 partial pressure from 40 to 56 kPa positively affected final CH_4 content, succinic acid titre, yield and productivity as well as the CO_2 fixation rate, while also decreasing by-product formation. Fermentation parameters such as succinic acid yield, productivity and titer were highest when using 8.3:1 gas-liquid ratio at 56 kPa CO_2 partial pressure. However, the highest final CH_4

content (95.4%) was reached when using gas-liquid ratio 5:1. Results obtained for succinic acid yield and productivity using *A. succinogenes* at different CO_2 partial pressures are in agreement with what has been reported in other studies (Xi et al., 2011).

The system was able to reach 95.4% CH_4 content after biogas upgrading, which is similar purity as commercial biogas upgrading technologies deliver (Ryckebosch et al., 2011), but in addition to purified CH_4 also successfully produced succinic acid. While countries have different standards for purity of natural gas used for gas grid injection and vehicle fuel, purity of 95-98% CH_4 is a common target (Basu et al., 2010).

Table 1. Process performance at different CO_2 partial pressures and gas-liquid ratios.

	CO_2 partial pressure (kPa)					
	Biogas (60 % CH_4/40 % CO_2)				**CO_2 gas**	
	40	**56**	**40**	**56**	**101.325**	**140**
CO_2 solubility (mM)	9.15	16.7	9.15	16.7	23.16	31.97
Gas-liquid ratio	8.3:1	8.3:1	5:1	5:1	-	-
CO_2 fixation rate (L CO_2 L^{-1} d^{-1})	1.35	2.59	1.52	1.77	-	-
Final CH_4 content in biogas (% v v^{-1})	76.4	91.1	85.2	95.4	-	-
Succinic acid yield (g g^{-1})	0.60	0.62	0.56	0.63	0.62	0.69
Succinic acid productivity (g L^{-1} h^{-1})	0.53	0.60	0.53	0.56	0.73	0.80
Succinic acid titre (g L^{-1} ± SD)	12.85 ± 0.01	14.39 ± 0.09	12.74 ± 0.22	13.53 ± 0.09	17.53 ± 0.22	19.28 ± 0.24
Acetic acid titre (g L^{-1} ± SD)	5.64 ± 0.09	5.10 ± 0.04	6.47 ± 0.45	5.61 ± 0.50	6.13 ± 0.14	5.58 ± 0.13
Formic acid titre (g L^{-1} ± SD)	3.86 ± 0.22	3.55 ± 0.02	5.16 ± 0.34	4.35 ± 0.48	4.32± 0.27	3.93 ± 0.24

4 Case study of a proposed biorefinery concept

Macroalgae biomass has been identified as a very promising feedstock for biorefinery (Kraan, 2010; van Hal et al., 2014). In this project, based on results from succinic acid fermentation and biogas production from seaweed species *L. digitata* presented in Paper VI, a more comprehensive biorefinery concept is proposed (Figure 9). While the two products were the main outcomes of that biorefinery study, there was observed a clear potential in producing numerous other products, mainly from the post hydrolysis solid residue (PHSR). Paper VI was the first of its kind were it is shown that removal of carbohydrates from seaweed biomass, results in up-concentration of other chemical constituents found in PHSR.

Carbohydrates found in hydrolysate (liquid fraction) are fermented into succinic acid as in Papers II, IV and VI, while the source of CO_2 for the fermentation comes from biogas (red arrows in Figure 9) as done in Paper III. Purified methane (biomethane) is thereby produced, along with succinic acid. CO_2 produced from other processes within the biorefinery could also be consumed within this process. The first obvious use for PHSR would be feed, since 352% higher protein content and 858% higher lipid content was seen when comparing PHSR to the original seaweed material. The increased protein and lipid content, opens up options for other application for the PHSR material. Through solvent extraction, commonly using a mixture of chloroform and methanol (Kumari et al., 2011), lipids can be extracted from PHSR. The extracted lipids can thereafter undergo a separation step where valuable and essential ω-3 and ω-6 (constitute >30% of total long chain fatty acids, LCFAs; content of the lipids in PHSR) are purified. Other LCFAs such as MUFAs and SFAs, which constitute 59% of total LCFA content in PHSR, are known to be good substrate for biodiesel production (Olmstead et al., 2013), and therefore biodiesel could also be a product in the proposed biorefinery. Following lipid extraction from PHSR, protein could be extracted e.g. through acid/alkaline coagulation (Dale et al., 2009). The residue that would be obtained after all extraction steps could then be used for anaerobic digestion to produce additional biogas. Additional products that could effectively be produced in this biorefinery concept are e.g. feed and fertilizer.

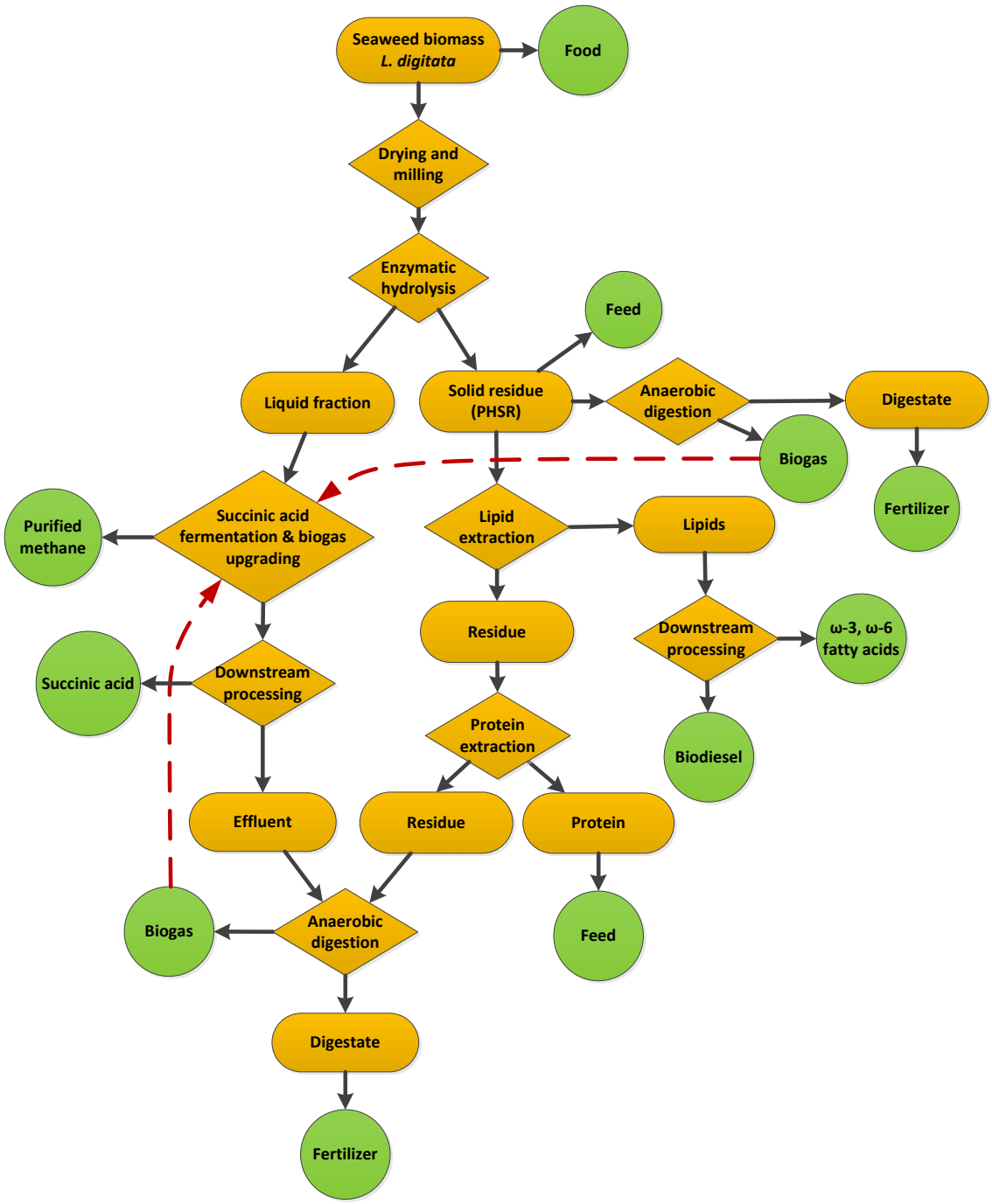

Figure 9. Process flow of a proposed seaweed biorefinery.

By including biological processes such as anaerobic digestion and succinic acid fermentation in the biorefinery concept, minimum waste and CO_2 emissions could be achieved. Compared to other studies also acknowledging the potential of macroalgae with respect to biorefinery applications (van Hal et al., 2014), the biorefinery concept proposed in Figure 9 includes a larger product portfolio. Having succinic acid as a key commodity in the biorefinery product portfolio introduces CO_2 fixation, which is likely to significantly

impact the sustainability of the overall concept. Also, as result of the CO_2 fixation, purified methane has much increased energy density as well as additional applications compared to biogas.

It can be argued that other types of biorefineries (Lignocellulosic feedstock biorefinery, whole-crop biorefinery and green biorefinery) could utilize similar processes as shown in Figure 9, with an added pretreatment step upstream. However, due to the presence of lignin in land-based biomasses, other processes where lignin is extracted, and then directly combusted or utilized for chemical synthesis are necessary.

5 Conclusions

This thesis focused on biofuels and succinic acid production from both land based lignocellulose and aquatic macroalgal biomass within a biorefinery concept. What other commodities can potentially be produced within the biorefinery was also investigated. Various pretreatment and saccharification techniques were utilized, followed by anaerobic digestion, ethanol- or succinic acid fermentation, using different microorganisms. The major contributions resulting from these studies are summarized below.

- The chemical composition of Jerusalem artichoke varied largely between harvesting time, where lignocellulosic part of this plant showed relatively low cellulose content, compared to hemp and *L. digitata*.
- Chemical characterization of Jerusalem artichoke tubers, industrial hemp and macroalgae *L. digitata* showed that carbohydrates were present in high amounts (54-88% dry weight).
- Ethanol yields in the range of 74-92% of theoretical yield were observed when fermenting hemp hydrolysate, while ethanol concentrations amounted up to 10.0 g L^{-1}.
- Out of the thermochemical pretreatments investigated for hemp biomass, 3% H_2O_2 at 121°C for 1 h followed by enzymatic hydrolysis was most effective one, with an overall sugar yield of 73.5%.
- Jerusalem artichoke tubers, industrial hemp and *L. digitata* all showed considerable potential as feedstock for succinic acid production. The highest succinic acid titer, 47.4 g L^{-1}, was reached when fermenting Jerusalem artichoke hydrolysate, while the maximum succinic acid yield (86.5%) was reached when fermenting *L. digitata* hydrolysate.
- Energy recovery of post hydrolysis solid residue (PHSR) and fermentation broth effluent through anaerobic digestion corresponded to 298 and 285 NmL CH_4 g^{-1} VS_{added}, respectively.
- A novel biogas upgrading technology based on the CO_2 fixation abilities of *Actinobacillus succinogenes* 130Z was developed. The system was shown to be capable of simultaneously producing high purity CH_4 (≤95.4%) and succinic acid.
- A case study, proposing a macroalgae biorefinery concept, highlighted the potential of PHSR for production of additional products, such as ω-3 and ω-6 fatty acids, biodiesel, protein, feed, biogas and fertilizer, thereby diversifying the product portfolio.

6 Future perspectives

This project showed that high yielding unconventional biomasses can be utilized for the production of both biofuels and biochemicals. To further improve biomass utilization in the biorefinery concept additional research on the following points is suggested:

- Full utilization of PHSR as requirement for expanding the product portfolio in the biorefinery. However, additional information e.g. on lipid and protein extraction yields is still needed to determine which processes, and in what order they should be placed so that maximum value can be generated out of PHSR.
- Further development and optimization of the "simultaneous biogas upgrading and succinic acid production" technology is also required, so that it may be applied at larger scale.
- Metabolic engineering of *Actinobacillus succinogenes* to produce succinic acid at very high titer >100 g L^{-1}, with low, or even no formation of by-products.
- Life cycle assessment (LCA) and cost-benefit analysis could be conducted on the proposed macroalgae biorefinery concept, to get an idea about the carbon footprint and environmental sustainability of the overall process, as well as the economic benefits or drawbacks of the concept.

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8 Papers

I Gunnarsson, I.B., Svensson, S.-E., Johansson, E., Karakashev, D., Angelidaki, I., 2014. Potential of Jerusalem artichoke (*Helianthus tuberosus* L.) as a biorefinery crop. *Industrial Crops and Products* 56, 231–240.

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In this online version of the thesis, the papers are not included but can be obtained from electronic article databases e.g. via www.orbit.dtu.dk or on request from:

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